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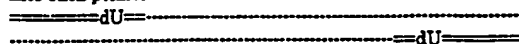
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(21) International Application Number: <b>PCT/IL98/00094</b> (22) International Filing Date: 26 February 1998 (26.02.98) (30) Priority Data: 120337                      27 February 1997 (27.02.97)      IL (71) Applicant (for all designated States except US): GESHER-ISRAEL ADVANCED BIOTECS (1996) LTD. [IL/IL]; P.O. Box 98, 90830 Bet Neqofa (IL). (72) Inventor; and (75) Inventor/Applicant (for US only): SHARON, Gil [IL/IL]; Cramim Street 48, 90805 Mevasseret Zion (IL). (74) Agents: LUZZATTO, Kfir et al.; Luzzatto & Luzzatto, P.O. Box 5352, 84152 Beer-Sheva (IL).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	

## (54) Title: METHOD FOR SIMULTANEOUS LIGATION OF MULTIPLE DNA FRAGMENTS

## (57) Abstract

A method is described for the simultaneous assembly of two or more primer extension generated, double stranded DNA fragments, into a construct, comprising the steps of: 1) designing the primers such that the fragments subsequently produced by PCR, have 3' termini of 15 nucleotides or more being complementary to a second and third fragment, both having 3' termini of 15 nucleotides or more, complementary to each terminal of first said fragment; 2) providing in each of said primers and subsequently in each of said fragments, at least one dU residue in place of at least one dT residue, being positioned at least 15 nucleotides from the 5' end of said primer and subsequent fragments; 3) adding to the mixture of fragments the enzyme UDG (Uracil DNA Glycosylase) and a compound such as N,N-dimethylethylenedimethylamine, to a-purinate and generate single strand nicks at said dU residues, respectively, thereby to generate a 3' single strand overhang on each fragment to be joined thereto, being complementary to the other 3' single strand overhangs of second, third or more aforementioned fragments, and 4) allowing for all fragments thus generated to anneal into a single and stable DNA construct.

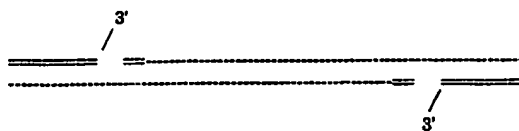
A. PCR fragment with a single dU residue that had been incorporated into each primer



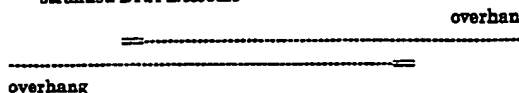
B. UDG a-purinates the dU residues



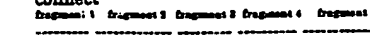
C. N,N dimethylethylenediamine nicks the a-purinated residues



D. Heating disconnects the nicked oligonucleotides from the double-stranded DNA molecule



E. Slow cooling allows fragments with complementary termini to interconnect



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## METHOD FOR SIMULTANEOUS LIGATION OF MULTIPLE DNA FRAGMENTS

**Field of the Invention**

The present invention concerns an improved method for joining a number of DNA fragments into a single, complex DNA construct in a desired predetermined directional manner in which method the actual joining of the DNA fragments is essentially a single step. More specifically, the present invention concerns an improved method for combining DNA fragments having regulatory function, such as, for example, promoters and the like, and DNA fragments encoding various proteins, such as, for example, enzymes (such as polyketide synthases), cytokines, hormones and the like, into a single construct for the purposes of cloning and expression of such a construct in a simple and rapid procedure, by way of which the various DNA fragments are connected in a predetermined direction and after joining of the fragments, there is no unwanted linker DNA between the joined fragments. Accordingly, the method of the present invention provides also for a seamless joining of DNA fragments.

**Background of the Invention**

Building DNA constructs is the core of genetic engineering. Building complex constructs requires time, money and highly skilled personnel. The construction is performed by fusing together specific DNA fragments in a desired way. The state of the art, as concerns cloning DNA fragments, has been published in a very large number of books, articles, patent applications, patents, and the like, and is usually readily available and known to all of skill in the art. For example, a comprehensive account of DNA cloning procedures is provided in the three volume text by Sambrook et al. (1989) entitled "Molecular Cloning - a Laboratory Manual", 2nd Edition, Cold Spring Harbor Laboratory Press. This extensive account of the prior art techniques for the combination of DNA fragments, cloning and expression thereof, is included herein by reference, in its entirety.

In accordance with the prior art, each of the DNA fragments is initially cleaved from larger DNA entities (DNA from plasmids, cDNA, genomic DNA) with enzymes called restriction enzymes. The desired fragments are then covalently connected together by use of an enzyme called ligase.

A given restriction enzyme is able to cleave DNA at a specific short sequence known as a restriction site. With more than two hundred different restriction enzymes which are commercially available, restriction sites situated at random up and downstream of a desired gene (or any other sequence of choice on a given DNA entity), can be identified and then cleaved by the respective enzymes. The DNA sequences which constitute restriction sites are mostly palindromic, between four to eight base pair long. Most enzymes cleave the DNA within the restriction site, leaving either "blunt" or "staggered" ends, depending on the specific enzyme. DNA with a "staggered" end has a short stretch (also known as "overhang") of single-stranded DNA between two to four bases long.

The enzyme ligase can connect, or ligate, two blunt ends of two DNA fragments (each fragment is a separate molecule) and form one longer fragment (longer molecule). Such a ligation is extremely inefficient. Ligase can also ligate two molecules with staggered ends if the overhangs of these two molecules are complementary to each other. In fact, matching overhangs greatly improve ligation between molecules: the single-stranded DNA on one molecule has an affinity to the complementary single-stranded DNA on the other molecule. The staggered ends, also known as "sticky ends", or overhangs, form non-covalent connection with one another via hydrogen bonds. Since the overhangs produced by restriction enzymes are short, these connections are weak and unstable. Nevertheless, they rightly align the molecules long enough as to assist ligase in performing its task.

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Sticky-end overhangs produced by the majority of the restriction enzymes consist of an overhang of two or four nucleotides. In theory, only fragments containing matching complementary overhangs can be connected to one another by ligase. In practice, illegitimate connections are a common occurrence. Due to the low affinity between such short single strand overhangs and consequently, due to the unstable nature of the connection between them, legitimate ligation is an inefficient process yielding a low amount of desired product. Furthermore, the palindromic nature of the sticky ends always results in undesired by-products such as "head-to-head" connections between identical molecules which further reduce the amount of the desired product.

Connecting DNA fragments with non-matching ends can be carried out by either blunting the ends by special enzymes or by adding very short, artificial DNA molecules called "linkers". These molecules are specifically designed to have an overhang that would match one fragment on one of their sides and another overhang, on the other side, that would match the other fragment. The addition of linkers further reduces the amount of the desired product.

Because of the inefficiency of the process, connecting more than two fragments at once is avoided if possible, and the building of DNA constructs is done one step at a time. Each step consists of several stages: first, desired DNA fragments are cut by restriction enzymes from larger molecules. Next, two DNA fragments are ligated to each other. Since the amount of the desired fragment is low, it has to be amplified, usually by transfecting cells of choice, such as, for example, bacterial cells. In order to do so, the product has to be circular DNA and has to contain certain components that will allow its amplification in bacteria. The third stage is therefore transformation of the DNA product into bacterial cells. Because of the high background of undesired product, a fourth, verification, stage has to be carried out. In this stage, the DNA from various bacterial clones

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is purified and tested in order to distinguish between the desired product and all the others. Only afterwards can one proceed to the next step. The construction of a complex DNA molecule requires numerous such steps. In terms of time, the construction of sophisticated molecules may take anywhere between several weeks to several months, and sometimes it is not achieved at all.

Although the affinity between complementary overhangs of two or four nucleotides is low, both the affinity between complementary overhangs and the stability of the hydrogen bonds, once formed, greatly increase when the overhangs are longer. Several methods for connecting fragments employing overhangs of longer than 4 bases have been described.

One prior art method uses single strand extensions created by adding nucleotides at the 3' end of a DNA strand in a template-independent fashion (Roychoudhury, R. Gene Amplif Anal. 2:41-83, 1981). The enzyme used in this method, terminal transferase, will incorporate nucleotides at the 3' hydroxyl terminus of a double-stranded DNA fragment, thus creating a single-stranded tail. Since the enzyme uses the nucleotides randomly, the only way to ensure that the single-stranded tail will be complementary to a corresponding overhang created on a second DNA molecule, is to provide for each extension only one of the four nucleotides. The overhangs created with this method must therefore be homopolymeric, so that only four types of overhangs can be used, corresponding to the residues dA, dC, dG or dT. Since the overhangs created on both termini of a DNA fragment must be identical, cloning with this method is directionless and can only involve two fragments that are connected to each other at both ends, forming a circular molecule. Furthermore, the length of the overhangs cannot be specifically controlled. Finally, the method necessarily introduces an unwanted stretch of nucleotides into the final construct, the length of which cannot be

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determined exactly, making the method unsuitable for the purpose of cloning into vectors where the reading frame must be preserved.

Various cloning kits sold in the market are based on the fact that the hydrogen bonds between overhangs 12 nucleotides long are stable enough to make the addition of ligase prior to transformation into bacteria unnecessary. The hydrogen bound fragments remain attached to one another during the transformation procedure and then become covalently bound by the bacterial ligation machinery. In fact, in a number of recent issued U.S. patents and a published PCT patent application: U.S. 5,137,814; U.S. 5,229,283; U.S. 5,334,515; and WO/18175 (PCT/US93/01965), all assigned to Life Technologies, Inc., U.S., there have been described methods for inserting DNA fragments of choice into a DNA vector of choice by way of generation of such 12 nucleotide long complementary overhangs, as well as ways of altering the nucleotide sequence of such DNA fragments at the time of their preparation and insertion into the vectors.

The above-mentioned U.S. patents and PCT application assigned to Life Technologies, Inc., also describe the use of so-called exo-sample nucleotides such as, for example, dU for the purposes of generating the desired complementary overhangs on the vector and the fragment to be inserted therein. Hence, these patents and application are also incorporated herein in their entirety by reference for all matters concerning the use of exo-sample nucleotides and the various associated polymerase chain reaction (PCR) procedures and the construction of single-stranded DNA primers, including such exo-sample nucleotides, to generate the fragment of choice to be inserted into the vector.

However, the above-mentioned prior art as a whole, and the specific disclosures according to the above-mentioned U.S. patents and PCT application, all have drawbacks when it is desired to use such known

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technology to combine two or more DNA fragments together. Of these drawbacks, one of the main ones is the fact that for the generation of the complementary overhangs, usually repeats of three nucleotides are employed, each repeat containing a single EXO-sample nucleotide, in the primer sequence to generate the desired fragment. As a result, the generated fragment will have at its terminal ends an additional segment of DNA corresponding to these repeats. This additional segment of DNA, also called "linker" DNA, has the length of the repeats. Subsequently, when the fragment is inserted into the vector, the two junction sites between the fragment and the vector will contain this extra linker DNA. Hence, if it was desired, for the purpose of expression of the fragment, to directly fuse the original fragment DNA to a desired sequence within the vector, by these prior art techniques such a direct fusion is not possible, because of the presence of the linker segment. Such a linker segment may therefore be detrimental to the proper expression of the fragment within the vector, and hence may have to be removed after the initial construction of the fragment within the vector, a procedure requiring an additional input of time and resources.

Similar methods have been described by Rashtchian et al. (Anal. Biochem. 206, p. 91-97, 1992), who described 12 nucleotide overhangs generated by uracil DNA glycosylase (UDG) to achieve high-efficiency cloning of single inserts into a vector. Using such complementary overhangs of only 12 nucleotides in length represents a further drawback of the above earlier methods, namely, such short overhangs often lead to a relatively low efficiency of joining between two fragments and hence, when three or more of such fragments are desired to be joined, these methods do not provide a useful yield of the desired products. Thus, using such 12 nucleotide overhangs with the prior art methods, it is likely that when it is desired to join three or more DNA molecules together, this would have to be done in a step-by-step process in which, at first, two molecules would be joined, the so-joined molecule then isolated and purified, and afterwards, the



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third fragment would be added thereto, and so on for any additional fragments to be joined. As a result, these prior art methods are also time-consuming and require a significant input of resources.

One of the aims of the present invention is to provide a method by which two or more fragments may be joined together in a specific predetermined directional manner, very efficiently, in which the joining step is essentially a single step and in which the junction sites between the various fragments are seamless, namely, do not contain any linker DNA segments to provide a fully in-phase joining of one fragment to the next.

In a copending application of the same applicant herein, filed together with the present application and identified as Attorney Docket 4190/96, (Israeli Patent Application No. 120338) there is described a method for joining DNA fragments in an efficient manner, by way of a rapid procedure, and this by providing long terminal overhangs of at least 15 nucleotides on each of the fragments to be joined. It is another purpose of the present invention to provide a method for joining three or more DNA fragments in an efficient manner, and at the same time ensuring that there is only complementarity between the terminal overhangs of any two fragments which are to be joined together, ensuring that only the predetermined desired order of joining between the fragments is achieved.

Other aims and aspects of the present invention will be readily apparent from the foregoing disclosure.

It should be noted that all of the various terms, procedures and the like, used herein throughout, unless otherwise indicated, are all well known terms in the art, known by all of average skill in the art. Thus, for example, terms such as nucleotides, primers, PCR, and the like, are readily known to all of skill in the art and are well defined in all standard texts and publications, for example, the above-noted Sambrook et al. The

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above-noted U.S. patents and PCT application, incorporated herein by reference, are less known. The term "exo-sample nucleotides", of which dU is an example, as used herein throughout, is a relatively new term of the art, but is well defined and described in the above-noted reference U.S. patents and PCT application.

### Summary of the Invention

In accordance with the present invention, there is provided an improved method for combining two or more DNA fragments together into a multi-DNA fragment assembly, which overcomes the above-mentioned drawbacks of the prior art. By the method of the present invention, the DNA fragments are joined in an essentially single-step joining reaction in a desired, predetermined order and in a seamless fashion.

Accordingly, the present invention provides a method for the simultaneous multi-DNA fragment assembly of two or more double-stranded DNA fragments produced by a primer extension reaction, particularly the polymerase chain reaction (PCR), comprising the steps of:

- (a) providing for each DNA fragment to be joined to a second DNA fragment and to a third DNA fragment, a pair of primers for the primer extension procedure thereof, wherein one of said two primers has a portion which is complementary to one terminal portion of the strand of said fragment, and the second of said two primers has a portion which is complementary to the other terminal portion of the strand of said fragment, and wherein the second portion of at least one of the said primers is complementary to the terminal portion of a different fragment to be joined to said first fragment in a specific positioned relationship;
- (b) providing, in each of said primers, at least one dU residue or the like in place of at least one dT residue of the original primer sequence, the first of said at least one dU residue(s) being positioned at least 15 nucleotides from the 5' end of said primer;

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- (c) producing each said DNA fragment by the primer extension reaction procedure;
- (d) carrying out in any suitable order the steps of: 1. mixing the produced DNA fragments 2. adding the enzyme UDG to apurinate said dU residue and a second enzyme or a compound such as N,N-dimethyl ethylenediamine to generate single-strand nicks at said apurinated dU residue, said UDG and second enzyme or compound being in amounts sufficient thereby to generate at the end of substantially all fragments a 3' single strand overhang which is complementary to another 3' single strand overhang or another fragment to be joined thereto; and
- (e) providing suitable conditions and allowing sufficient time for the specific joining of said fragments to generate a multi-DNA fragment assembled product.

Of course, if two fragments are to be joined to a circular construct, then the above termini of said second and third DNA fragments are termini of the same fragment. Furthermore, it will be readily appreciated by all of skill in the art that it is possible, by the above method of the invention, to join a fragment at one end only, i.e., it is not necessary to join both ends of any or all of the above fragments.

The invention also provides the above method wherein the DNA mixing step is carried out before the UDG enzyme addition step. The invention further provides the above method wherein the UDG enzyme addition step is carried out before the DNA mixing step.

Still further, the invention provides the above method wherein step (e) includes the removal of the nicked oligonucleotides from the reaction.

One embodiment of the above method of the invention is a method wherein the said 3' complementary overhangs on said DNA fragments are

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generated by the addition to said fragments, the enzyme UDG to apurinate said dU residue and a second enzyme selected from endonuclease III (Endo III) or endonuclease IV (Endo IV) to generate single-stranded nicks of said apurinated dU residue to provide said 3' overhangs.

Another embodiment of the above method of the present invention is a method wherein said 3' complementary overhangs of said DNA fragments are generated by the addition to said fragments, the enzyme UDG to apurinate said dU residue and the compound N,N-dimethyl ethylenediamine to generate single-stranded nicks at said a-purinated dU residue to provide said 3' overhangs (for the role of this, and other chemical reagents in forming nicks, see McHugh and Knowland. (1995) Nucleic Acid Res., 23, 1664-1670).

The joining of the fragments via the 3' complementary overhangs is facilitated by:

(a) heating the reaction mixture to a temperature suitable to allow dissociation of the oligonucleotides at the termini of the fragment after said apurination of the dU residue and generation of nicks at the apurinated dU residue, and incubating said mixture;

(b) keeping the mixture of (a) at a temperature suitable to maximize correct connections between complementary overhangs; and

(c) optionally, adding ligase to said mixture to facilitate covalent joining of the DNA strands.

In accordance with any of the above methods and embodiments according to the present invention, the DNA fragments to be joined are selected from two or more DNA fragments having regulatory function such as, for example, promoters, enhancers, terminators, ribosome binding sites and the like; and DNA fragments encoding proteins such as, for example,

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enzymes (such as citrate synthases, polyketide synthases, and succinyl-CoA-synthetase), cytokines, hormones and the like.

Furthermore, in accordance with any of the methods or embodiments of the present invention as noted above, there is also provided a method wherein one or more of the DNA fragments to be joined is a mutant fragment having been subjected to site directed mutagenesis during its preparation. Thus, for example, if it is desired to join a promoter carried by one DNA fragment to another DNA fragment encoding the protein of choice, it is possible to produce one or more site directed mutations in the promoter sequence and this by introducing into the primers made to synthesize this promoter sequence one or more specific site-directed mutations to provide a mutated promoter sequence, or similarly, by introducing mutations into the primers made to synthesize the protein sequence, it is possible to generate a site-directed mutated protein sequence. This is particularly useful if, for example, it is desired to provide a promoter from a particular source but in which its activity is either increased or decreased with respect to the naturally occurring promoter or, likewise, if it is desired to alter the biological activity of the protein as compared to its naturally occurring form. Thus, by virtue of the present invention, it is possible not only to join fragments of choice together, but at the time of preparing such fragments, it is possible to also mutate the sequences of the fragments of choice. In addition, it is also possible to generate random mutations in defined sequences by employing mutagenic PCR conditions on at least one of the fragments which are produced.

According to the present invention, there is also provided the above method, wherein at least some of said primers for the primer extension production of said DNA fragments are biotinylated at their 5' ends, and wherein when said enzyme UDG and said second enzyme or compound are added to said mixture of fragments, there is also added streptavidin, either

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free or bound to beads, whereby single-stranded oligonucleotides containing at their 5' end a biotin moiety generated following the formation of said single-strand nicks at said apurinated dU residues are bound via a biotin-streptavidin connection and are effectively removed from the joining reaction.

The present invention also provides a primer for use in any of the above-mentioned methods or embodiments thereof of the present invention, comprising at least one dU residue in place of at least one dT residue, the first of said at least one dU residue(s) being positioned at least 15 nucleotides from the 5' end of the primer. In view of the above-mentioned, there is also provided, as an embodiment of the primer of the invention, a primer wherein the primer is biotinylated at its 5' end.

The present invention also provides an assembled DNA construct whenever prepared by any one of the methods or embodiments thereof as set forth hereinabove, and wherein said construct has been assembled from the joining together of a plurality of DNA fragments according to the invention.

Still further, the invention provides a DNA fragment comprising an overhang of at least 15 nucleotides or an end portion suitable to be converted into such an overhang. The invention also provides said DNA fragment, for use in the above method according to the invention.

Illustrative, but non-limitative, examples of the above assembled DNA construct of the invention include constructs which have been assembled by the joining together of three DNA fragments; constructs which have been assembled by the joining together of four DNA fragments; constructs which have been assembled by the joining together of five DNA fragments, and constructs which have been assembled by the joining together of eight and more DNA fragments. Furthermore, other examples of the assembled DNA constructs of the invention include constructs as indicated above,

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which are in the form of a linear DNA molecule or which are in the form of a closed circular DNA molecule.

Other aspects and embodiments of the present invention will be readily apparent from the following detailed description of the invention.

### **Brief Description of the Drawings**

- Fig. 1 (A-E) is a schematic flow-diagram depicting one embodiment of the preparation of the fragments, their treatment and their joining, in accordance with the method of the invention, as detailed in the Examples. == depicts the primers, ---- depicts a single strand of the DNA fragment and /\_\_\ depicts the a-purinated site.

- Fig. 2 is a schematic illustration of a plasmid prepared in accordance with the method of the present invention, as detailed in Example 1 and 2, wherein the open bars depict the Tet fragment which may be synthesized as a single fragment from primers SEQ. ID NO. 1 (also designated 31162) and SEQ. ID NO. 5 (also designated 30402), or in the form of two subfragments, TetA and TetB from primers SEQ. ID NO. 1 and SEQ. ID NO. 4 (also designated 27341), and SEQ. ID NO. 3 (also designated 25595) and SEQ. ID NO. 5, respectively; the dark bar depicts the Amp + ColE1-ORI fragment, which is synthesized as a single fragment from primers SEQ. ID NO. 8 (also designated 27342) and SEQ. ID NO. 6 (also designated 3885); and wherein the hatched bar represents the Cm fragment, which may be produced as a single fragment from primers SEQ. ID NO. 2 (also designated 3597) and SEQ. ID NO. 7 (also designated 4144), or as two separate subfragments, CmA and CmB from primers SEQ. ID NO. 2 and SEQ. ID NO. 9 (also designated 27343), and SEQ. ID NO. 10 (also designated 25596) and SEQ. ID NO. 7, respectively, and wherein the primers are indicated in the figure with arrows denoting their direction of synthesis, the arrowheads being the 3' end of the primer.

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Fig. 3 is a schematic illustration of a plasmid constructed out of eight fragments in accordance with the method of the present invention, as detailed in example 3.

### Detailed Description of the Invention

The present invention concerns an improved method for combining two or more DNA fragments together into a single DNA construct, by which method the fragments are joined in an essentially single-step joining reaction, in a desired, predetermined order and in a seamless fashion, namely, no linker DNA is inserted between the joined fragments.

In the present invention, each primer is synthesized by standard, automated single-stranded (oligonucleotide) DNA synthesis and has two parts. A 3' part being complementary to the fragment to be produced, and a 5' part complementary to the terminal portion of the strand of another fragment to be specifically joined to this first fragment. Thus, the primers, in accordance with the present invention, essentially correspond to the desired predetermined junction region at those ends of the two fragments to be joined. In this way, for each fragment generated, a unique pair of primers is used.

In the above primers, during their synthesis there is incorporated at least one dU residue in place of at least one dT residue of the original sequence of the fragment, this dU residue being placed at least 15 nucleotides from the 5' end of the primer. In this way, as is fully detailed below, when the so-generated fragment is to be joined to the other so-generated fragments, upon addition of the enzyme UDG and the compound N,N-dimethyl ethylenediamine, or another enzyme which is the functional equivalent of this compound, at the ends of each fragment there is generated a 3' single strand overhang which is complementary to only one other such overhang present only on the fragment to be joined, to ensure that only the right fragments will join.



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According to the present invention, the use of dU and the associated UDG and N,N-dimethyl ethylenediamine for the generation of the specific overhangs, is the preferred choice, as all of these reagents are readily available at relatively low costs. However, any other suitable exo-sample nucleotides, and corresponding reagents to remove them and generate single-stranded overhangs as are known in the art may also be used in accordance with the present invention. See, for example, the above-referenced patents assigned to Life Technologies, Inc., in which there is described dU, UDG and various other exo-sample nucleotides and reagents for their removal.

One such example of an alternate procedure is the use of UDG to cause apurination of the dU residues in the PCR - generated DNA fragments followed by the use of a second enzyme, which may be either endonuclease III (Endo III) or endonuclease IV (Endo IV), instead of N,N-dimethyl ethylenediamine, to nick the DNA fragments at the apurinated dU residue and thereby to generate the desired 3' overhangs on the DNA fragments.

In accordance with the present invention, overhangs of at least 15 nucleotides long are generated to ensure stable and efficient joining between the various fragments. In fact, in a series of experiments in which overhangs of only 12 nucleotides was used, disclosed in a copending patent application of the same applicant filed on the same day as this application (Attorney Docket 4191/96, Israeli Patent application No. 120339), the specification of which is incorporated herein by reference, it was found that when using fragments with overhangs of 12 bases, only low efficiency joining between two fragments could be achieved, and no joining between three or more fragments into a single construct could be achieved. Hence, in accordance with the present invention, it has been found that such overhangs must be at least 15 nucleotides long to ensure the joining of two or more fragments together in an efficient manner.

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In accordance with the present invention, it is of course also possible to generate one or more site-specific mutations within the one or more fragments to be joined together and this by standard procedures, in which the various above-noted primers, when generated, are generated having a specific nucleotide substitution, deletion or addition at a selected site within the primer. Subsequently, the fragment generated from such a primer will have the pre-selected site-specific mutation. Likewise, it is also possible to generate regions of either high mutation rates or very low mutation rates by amplifying fragments using mutagenic PCR protocols or high-fidelity enzymes, respectively.

As regards the various DNA fragments to be joined in accordance with the method of the present invention, these fragments may encode any DNA molecule of choice. For example, the fragments may encode various regulatory sequences such as, for example, promoters, enhancers, terminators or the like. The fragments may also encode various proteins having various biological activity of pharmaceutical or veterinary importance, for example, various metabolic enzymes (such as polyketide synthase), hormones, cytokines, and the like. Hence, it is possible to generate, in accordance with the present invention, new chimeric promoters having improved or decreased activity and this by joining two fragments encoding parts of promoters from various sources. Likewise, it is possible to generate a wide range of chimeric structural and regulatory proteins, for example, chimeric cytokine molecules, receptors, enzymes and the like, of improved or other desired biological activity, by combining fragments encoding different domains of such molecules from different sources. Likewise, a multi-fragment assembly may be devised in which a new desired promoter is directly connected to a new desired DNA molecule encoding a biologically active protein in a single new construct for the purposes of enhanced expression of this new desired protein, then the construct is used to transfect/transform suitable cells of any organism of

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choice, for example, prokaryotic or eukaryotic cells such as bacterial or yeast cells, respectively, or mammalian, insect or any other eukaryotic cells. Likewise, such a multifragment assembly can also be in the form of a modified bacterial or animal virus carrying one or more genes of choice for the purposes of infecting prokaryotic or eukaryotic cells of choice, and thereby introducing into these cells the gene(s) of choice.

In accordance with the present invention, the PCR procedure is essentially the well known, now standard, procedure, which may be augmented by using recently described new high-fidelity DNA Polymerase enzymes, as well as newly developed, improved automated machinery for this purpose. Hence, any PCR procedure and reagents for use therewith may be utilized in accordance with the present invention to generate the very specific fragments which are to be combined together.

Hence, in accordance with the present invention, one may readily prepare a DNA construct of choice, namely, a "custom-made" DNA construct in which any desired DNA fragment encoding any desired structural or regulatory function, can be joined in a seamless fashion to other such DNA fragments. As the procedure in accordance with the present invention is both rapid and simple to perform, it is therefore possible to prepare any DNA construct of choice, be it a linear DNA molecule for insertion into cells directly by known techniques, or a circular DNA molecule to be used as a vector for transfecting/transforming cells of choice, or a linear construct for insertion into another vector of choice, and any other such purpose readily apparent to any of skill in the art. The list of possible constructs which may be prepared in accordance with the present invention is essentially endless, as the only limitation on the preparation of constructs in accordance with the present invention is the availability of sequence information for the ends of each of the fragments to be joined.

The present invention will now be described in more detail in the following

non-limiting examples and the accompanying drawings:

### The General Procedure

The fragments which are to be attached to each other in a directional fashion are preferably prepared by utilizing the well-established Polymerase Chain Reaction (PCR) procedure, this being a standard procedure of the art. This provides for suitable amounts of the fragments.

In this way, the desired fragments of the original DNA are greatly amplified and by virtue of the use of pre-selected specific primers in the PCR procedure, the 5' and 3' ends (termini) of the PCR-prepared fragments will have the desired pre-selected sequences which will ultimately provide for the directional attachment of the fragments to yield the DNA molecule of choice, in which all of the fragments have been attached to each other in the pre-selected order.

Furthermore, by virtue of the necessity to use pre-selected primers in the PCR procedure, this necessity is also convenient for the purposes of the present invention, namely, into these primers, one or more dU residues may be incorporated in place of one or more dT residues, these one or more dU residues being the sites at which further specific treatment will provide for each of the various fragments having the desired single-stranded DNA overhangs (cohesive ends) that will be complementary only to the overhangs on the other fragments to which each fragment is to be connected, thereby ensuring the directionality of the connection.

In accordance with the present invention, it is preferred to use more than one such dU insertion into each primer in those sites of the primer that it is desired to treat further following the PCR procedure. However, it must be noted that the number of dU insertions is entirely dependent upon the chosen (predetermined) primer sequence, not all primer sequences of

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choice will necessarily have many dT residues for replacement by dU residues. Further, it is also desirable to have such dU replacements that are spread out along the length of the primer, but here too, the number and spread of dU replacements is dependent upon the primer sequences of choice. In any event, at least one dU insertion in place of one dT residue is essential in each primer sequence to ensure the successful operation of the method of the invention. According to one embodiment of the present invention, following the PCR procedure, all of the fragments are mixed together and subjected to the preferred treatment with UDG and N,N-dimethyl ethylenediamine, which results in the apurination of the dU residues, followed by cleavage (nick) at the 3' side of each apurinated residue to create a single strand nick. After this treatment, the temperature is raised to 75°C, resulting in the denaturation and disconnection of the double-stranded structures in between dU residues and up to the dU residue most distal from the 5' end of the primer (the most 3' dU residue), or when only one dU residue was inserted, then the denaturation and disconnection is up to the position of this dU residue, thereby yielding fragments having long specific 3' overhangs. These 3' overhangs are specific both as regards their sequence, and as regards their length, the length being determined by the original placement of the most distal (most 3') dU residue in the primer. In the present invention, overhangs of about 15 nucleotides and longer are used.

The above predetermined annealing can be done by a slow cooling, (starting from 75°C and down to about 20-30°C), which facilitates specific and directional annealing of the different fragments to each other, by virtue of specific connections between the complementary 3' overhangs of the neighboring fragments. Alternatively, the annealing of the fragment can be done at 37°C or at any temperature desired when more than one dU residue is inserted into the primer and the inserted dU residues are spaced along the primer, their subsequent removal results in the generation of very short released oligonucleotides which compete less

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successfully for annealing to the aforesaid strand (to which they were originally annealed) with the complementary overhangs of the fragments to be joined. Further, at the temperatures at which the various fragments join each other by annealing of their complementary 3' overhanging portions, the various released oligonucleotides are less efficient in reannealing, the shorter the oligonucleotide, the less the annealing efficiency. Most preferably, the oligonucleotides that are generated 3' to the apurinated dU residue or residues are removed from the mixture. Two examples of how to do it are included herein, one using biotin (Example 2), the other using a different procedure (Example 3).

As a final step in the above procedure, ligase (e.g., T<sub>4</sub> ligase; of course any ligase, other than T<sub>4</sub> ligase, may be used) may be added to covalently connect all of the fragments and thereby complete the formation of a single DNA molecule without any nicks. This ligase step is optional in view of the fact that by the preferred use of long overhangs at the termini of the fragments, the connection between the matching complementary overhangs, i.e., the hydrogen bonding therebetween, is of such a nature that even without the final covalent connection with a ligase, the hydrogen bonding is strong enough to hold all of the fragments together. As will be described below, fragments combined in this fashion remain intact in a linear form even when subjected to gel electrophoresis, or when fragments are so connected to form a circularized DNA molecule, such a circularized molecule is structurally stable enough to be used to transfect cells to provide transformed cells containing this circularized molecule, which in these cells become ligated by the endogenous cellular ligases.

For the purpose of simplicity, it should be noted that in the above general procedure, when, for example, it is desired to connect five fragments together to form a linear molecule, then, the specific primers used in the PCR step are designed in such a way that the ends that are to be connected to each other are homologous (i.e., have perfectly

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complementary 3' overhangs, once these overhangs have been generated as noted above and below). For example, if a linear DNA construct is to be made from five fragments numbered 1-5 and for each fragment, for the purposes of illustration, we designate the sense direction to be "left to right" such that when joined fragment number 1 will be the extreme left-hand side and fragment 5 will be the extreme right-hand side of the combined molecule (or by convention, the (+) sense strand will begin with its 5' terminus at the extreme 5' end of fragment 1 and end with its 3' terminus being at the extreme end of fragment number 5; and the (-) non-sense strand will have its extreme 5' end at the extreme end of fragment 5 and its 3' end at the extreme end of fragment number 1).

Hence, to make this construct of five fragments, the right terminus of fragment 1 should be homologous to the left terminus of fragment 2, the right terminus of fragment 2 should be homologous to the left terminus of fragment 3, the right terminus of fragment 3 should be homologous to the left terminus of fragment 4, and the right terminus of fragment 4 should be homologous to the left terminus of fragment 5. With such homology, once the 3' overhangs at the ends of each fragment are generated (as noted above and below), there will thus be perfect complementarity between the 3' overhang at the right terminus of fragment 1 and the 3' overhang at the left terminus of fragment 2 to provide for a specific directional joining of these two fragments by complementary interaction or joining between the two 3' overhangs, and likewise for the joining of the above-mentioned other termini of the various fragments to be joined. In such a construct, therefore, the left terminus of fragment 1 and the right terminus of fragment 5, i.e., the extreme ends of the molecule, should not have any homology to each other or to any of the other left or right termini of all of the various fragments, in this way ensuring that the so-produced molecule will be linear and will not be capable of circularization or undergoing any other inter-fragment interactions which would disturb the 1-2-3-4-5 desired configuration to be formed.

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However, when it is desired to make such a molecule which is circular, then of course the right terminus of fragment 5 should be homologous to the left terminus of fragment 1, thereby ensuring that circularization occurs only by connection between fragment 5 and fragment 1. As also arises from the above-mentioned, all of the left and right termini of each of the fragments which are not designed to be connected must be non-homologous, this condition being relatively easy to meet in view of the standard technology available for the automated synthesis of primers, the sequence of which is easy to determine.

Thus, it is apparent from the above general procedure that the method of the present invention is essentially a single-step procedure in which all of the reaction components are present in a single reaction vessel in which all of the various reactions and treatments are carried out, thereby greatly simplifying the overall process and providing an end-product that is essentially only the desired product which can be readily obtained and further utilized.

It should of course be noted that the initial PCR amplification of the fragments which it is desired to combine should preferably be carried out in separate reaction vessels for each specific fragment to ensure the fidelity of the PCR products.

However, this requirement for separate PCR amplifications of the selected sequences is now also a simple laboratory procedure in view of the widely available automated apparatuses for carrying out PCR procedures, which can handle a large number of separate samples at the same time. Also, as mentioned above, the production of the desired PCR primers which will carry the preferred more than one dU residue, or at least a single dU residue is also carried out by automated machinery which allows for the generation of large numbers of primers of high fidelity and purity



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simultaneously in a very short period of time (for example, there are machines which can simultaneously produce 96 different primers at a cost of only about 10-30 cents per base per primer). Hence, it is possible to readily produce almost any desired primer carrying the preferred more than one or at least single dU residue in the preferred positions or position within the primer so as to ultimately result in an at least 15 nucleotide overhang and preferably about 20 or more nucleotide overhang at the ends of the fragments to be joined.

A schematic representation of the above-mentioned general procedure is set forth in Fig. 1. In parts A-D, there is illustrated how a PCR fragment having only a single dU residue (for simplicity of illustration) may be treated with UDG and N,N-dimethyl ethylenediamine to yield a PCR fragment with 3' overhangs at its two termini. In parts A-B of Fig. 1 there is shown schematically the preferred position of the dU residue in the end-product of the PCR procedure, i.e., the desired PCR-produced fragment which has incorporated at its terminal ends the sequence defined by the two primers used in its preparation, and in which the dU residue is inserted distal from the terminal ends of the fragment so that once it is removed by the treatment with UDG and N,N-dimethyl ethylenediamine, schematically shown in parts C and D of Fig. 1, large single-stranded terminal overhangs will be formed, these preferably being at least 15 nucleotides in length, and more preferably about 20 or more nucleotides in length. When the primers are prepared it is of importance to make the replacement of the dT residue by the dU residue at a site in the primer which will be about 20 base pairs (bp) distal to the 5' end of the sequence of the desired fragment, so that when incorporated, this dU residue will be about 20 bp distal to the 5' terminus of the PCR end-product.

It should be noted that for the purposes of illustration in Fig. 1, parts A-D, there has been shown only the general outline of the UDG and

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N,N-dimethyl ethylenediamine treatment of dU-containing PCR fragments for a single exemplary fragment, all the other fragments that are to be joined together essentially having the general overall structural configurations with the dU inserted distal to the terminal ends of the fragments. In part E of Fig. 1, there is shown schematically the linear alignment of five fragments which are to be connected to each other in the predetermined order of fragments 1-5, this being facilitated by the homology at the ends of the fragments which are to be specifically joined to each other, namely, the so-called right-hand terminus of fragment 1 is homologous to the left-hand terminus of fragment 2, the right-hand terminus of fragment 2 is homologous to the left-hand terminus of fragment 3, and so on, so that only connections in the specific order of fragments 1-5 can be obtained by the specific complementary interactions between the complementary 3' overhangs. The length of the overhangs is playing a critical role in determining the strength of the hydrogen bonding between the complementary overhangs which will connect the various fragments and hence the overall high specificity of the order in which these fragments will be connected to each other.

### Materials and Methods

All of the fragments which were joined together, as set forth in the following examples, were first individually prepared by the PCR procedure using a commercially available PCR apparatus (Robocycler Gradient 96™, Stratagene, U.S.A.) according to the manufacturer's instructions.

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The concentrations of the reagents used in all of the PCR procedures, were as follows:

Reagent	Concentration	Volume in Reaction Vessel
DNA*(see below)	0.1 µg/µl	0.5 µl
Primer 1	10 pmol/µl	10 µl
Primer 2	10 pmol/µl	10 µl
dNTP**(see below)	2.5 mM (each)	16 µl
10X Buffer	--	20 µl
H <sub>2</sub> O	--	144 µl
TaqDNA Polymerase*** (see below)	5 U/µl	<u>0.8 µl</u>
		200 µl - Final Volume

The temperature regime for all of the PCR procedures was as follows, in accordance with the manufacturer's instructions:

94°C for 60 sec., 40°C for 80 sec., 72°C for 240 sec., this temperature regime being carried out for 30 cycles and then followed by a final 72°C for 600 sec. After the last 72°C incubation, the reaction vessel was cooled to 6°C.

\*The DNA which was used as the template in all of the following examples was either the plasmid pBR322 or the plasmid pACYC184, both commercially available and for both the full sequence and restriction maps are available. This will be described in more detail in Example 1 below.

\*\*dNTP is a mixture of dATP, dTTP, dCTP and dGTP, in equimolar amounts, all obtained from commercial suppliers and used in accordance with the manufacturer's instructions (Boehringer-Mannheim, Germany). It should be noted, as mentioned above, that during the PCR amplification of the fragments, only these normal nucleotides are employed and that dUTP is not inserted at all, this having been already inserted into the above-noted primer 1 and primer 2 prior to commencement of the PCR procedure.

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\*\*\*The Taq DNA polymerase was also obtained from a commercial supplier and used in accordance with the manufacturer's instructions (Boehringer-Mannheim, Germany).

Further, it should be noted that the above 10X Buffer is the usual buffer employed in the PCR procedure and is also purchased from a commercial supplier and used in accordance with the manufacturer's instructions (Boehringer-Mannheim, Germany).

### Example 1

#### Preparation of a circularized plasmid by the directional connection of 3-5 fragments constituting the plasmid

##### Overview

The plasmid to be constructed was designed to have three or more different regions, each to be prepared separately by PCR amplification and then joined in a specific directional fashion to provide a circularized plasmid as the end-product. This required the initial preparation of specific primers, the sequence of which was to provide the basis on which the various PCR fragments, constituting the various regions of the plasmid, would be able to combine with each other to ultimately connect all the regions in the specific directional manner. The sites within the primer sequences which would provide for the interconnection between the various PCR fragments were determined irrespective of any restriction enzyme sites or any other DNA sequence features at these connection sites in order to demonstrate that, in accordance with the method of the present invention, any DNA sequence at any particular site within a DNA molecule can be utilized as a connection site by preparing the necessary specific primers to provide for this connection site.

In Fig. 2, there is shown schematically the plasmid that was designed and produced by the method of the present invention. This plasmid carries

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three independent antibiotic resistance genes, for resistance to ampicillin (Amp gene, or hereinafter Amp); tetracycline (Tet<sup>r</sup> gene, or hereinafter Tet); and chloramphenicol (Cm<sup>r</sup> gene, or hereinafter Cm). The plasmid also carries the ColE1 origin of replication (ColE1-ORI), which in this specific instance is situated next to the Amp gene, thus these two entities constituting a single region of the plasmid. The Tet and Cm genes constitute additional separate regions. Such a plasmid is capable of being replicated in a host cell and will endow the host cell, successfully transformed therewith, with resistance to all three types of antibiotic. Accordingly, it is also possible to readily select for those host cells transformed by this plasmid by growing the transformed cells in the presence of one of the antibiotics and then to screen for the resistance to the other antibiotics. This therefore provides for a genetic analysis of the end-products of the construction procedure at the *in vivo* level. Further verification step was done by testing the plasmids in the transformed colonies by PCR, as detailed below.

To prepare the above plasmids, . number of constructions were made in which 3, 4 and 5 individual fragments were produced by PCR and then combined to yield the plasmids. Thus, when the plasmid was to be constructed from three fragments, the above-noted three regions of the plasmid were PCR synthesized using appropriate primers so that three PCR fragments together having all three regions would be obtained. When the plasmid was constructed from four fragments, other appropriate primers were used to yield four PCR fragments encompassing the three regions of the plasmid. Likewise when the plasmid was constructed from five fragments, other appropriate primers were used to yield five PCR fragments encompassing the three regions of the plasmid. The results (not shown) of these constructions using three and four fragments showed that the so-constructed plasmids were fully active *in vivo* when used to transfect host cells, there being obtained a large number of resulting transformed cells having resistance to all three antibiotics, this large

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number being indicative that the transformation efficiency of the so-constructed plasmid was high, and further, that the so-transformed cells actively expressed the antibiotic resistance genes carried by the plasmid.

For the purposes of exemplifying the method of the present invention, it has been chosen not to show the building of the simpler constructs and to demonstrate only the more difficult procedure for successfully combining five PCR fragments which together encompass the above three regions of the plasmid that was constructed and used to transform host cells with high efficiency.

For this five fragment construction, the following was carried out:

**(i) Preparation of the specific primers for the PCR amplification of the desired five fragments of the plasmid.**

As shown in Fig. 2, and as outlined above, it was desired to construct a circularized plasmid having three regions:

- (a) a Tet region;
- (b) an Amp + ColE1-ORI region; and
- (c) a Cm region.

These three regions were to be connected in a highly specific manner, namely, the Tet region was to be connected between two ends of Cm and Amp + ColE1-ORI regions, while at the other two ends, the Cm and Amp+ColE1-ORI regions were to be connected to each other. Thus, it was necessary to connect one end (upstream) of the Cm region to one end (upstream) of the Tet region, to connect the other end of the Tet region to one end (downstream) of the Amp + ColE1-ORI region, and to connect the other ends (downstream of Cm and the upstream end of Amp + ColE1-ORI region) to each other.

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To achieve this task, five separate fragments were designed and produced by PCR:

- (1) A large fragment of around 1700 bp which carries the Amp gene and the ColE1-ORI sequence;
- (2) Two fragments together constituting the Tet region being designated "Tet A" fragment of around 850 bp containing the upstream end of the Tet gene; and "Tet B" fragment of 600 bp containing the downstream end of the Tet gene; and
- (3) Two fragments together constituting the Cm region, designated "Cm A" fragment of around 430 bp containing the upstream end of the Cm gene; and "Cm B" fragment of around 270 bp containing the downstream end of the Cm gene.

For the production of these fragments, it was necessary to prepare specific primers. The above three regions of the plasmid to be constructed are well known, as they are derived from two commercially available plasmids, namely pBR322 and pACYC184 (New England Biolabs, U.S.A.). The full sequence and maps of the various regions of these plasmids are known. The full sequence of these plasmids can be accessed from GenBank database under accession Nos. J01749 and X06403, respectively. For the preparation of PCR fragments containing the Tet and Amp + ColE1-ORI regions the pBR322 sequence and plasmid (as a template) was utilized; and for the Cm region, the pACYC184 sequence and plasmid (as a template) was used.

In this way, using standard automated procedures to produce polynucleotide oligomers (Applied Biosystems, U.S.A.), the following primers were synthesized:

- (1) Upon a study of the sequences of the Cm gene provided with the commercially obtained pACYC184 plasmid and of the Tet gene provided with the commercially obtained pBR322 plasmid, it was

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decided that the desired region containing the junction between the Cm and Tet genes should be of the following sequence:

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                                junction point
5' CCATTTTAGC TTCCTT AGCTCCTGA↓TTCTCAT GTTT GACAGCTTATCATC
3' GGTAAAATCGAAGGAATCGAGGACT↑AAGAGTACAAACTGTCGAATAGTAG
<-----|----->
Cm upstream (5' region)      junction point      Tet upstream..(5') region

```

Hence, the primers which will provide the overlap between the Cm and Tet genes and allow their connection to each other were designed as follows:

(a) Primer designated SEQ. ID NO. 1, also designated 31162, as shown schematically in Fig. 2, having the sequence:

```

          5' A G C T C C T G A ↑ T T C T C A T G T T U G A C A G C T T A T C
sequence complementary      ↑      beginning of Tet 5' (sense) sequence
to the Cm end                junction point

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(b) Primer designated SEQ. ID NO. 2, also designated 3597, as shown schematically in Fig. 2, having the sequence:

```

          5' A A A C A T G A G A A ↑ T C A G G A G C U A A G G A A G C T A A A A T G
sequence complementary      ↑      beginning of Cm 5' (sense) sequence
to the Tet 5' (sense) end    junction point

```

As depicted schematically in Fig. 2, the above primer SEQ. ID NO. 1 will enable synthesis of the Tet gene from the upstream side, inclusive of the Tet A fragment, this synthesis being in the clockwise direction with respect to the completed plasmid shown in Fig. 2. At the same time, primer SEQ. ID NO. 1 has also an extension at its 5' end, as noted above, that is complementary to the Cm upstream region, to facilitate joining of the Tet and Cm genes.



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Likewise, the above primer SEQ. ID NO. 2 will enable synthesis of the Cm gene from the upstream side, inclusive of the CmA fragment, this synthesis being in the counterclockwise direction with respect to the completed plasmid shown in Fig. 2. At the same time, primer SEQ. ID NO. 2 also has an extension at its 5' end, as noted above, that is complementary to the Tet upstream region, to facilitate joining of the Cm and Tet genes.

It should also be noted that, by design, the two genes, Cm and Tet are to be joined at their 5' ends, i.e., when expressed they will be transcribed in opposite directions, this being apparent from the description above.

Furthermore, as arises from the positioning of the dU in the above primers SEQ. ID NO. 1 and SEQ. ID NO. 2, this was chosen so as to produce a large overlap (preferably greater than 15 nucleotides), namely, in the resulting PCR fragments produced with these primers, the dU is twenty nucleotides from the 5' end, and following UDG, N,N-dimethyl ethylenediamine treatment and heating, this entire twenty nucleotide single-stranded 5' fragment will be removed from both PCR fragments, leaving single-stranded 3' overhangs on the complementary fragments. This complementarity is unique (all the other primers for the other fragments having different sequences) to facilitate specific directional joining of the two fragments. As will be apparent to one of skill in the art from the above description and knowledge of the now standard PCR technology, use of the above primers will yield PCR fragments of the following 5'-end sequences containing the dU residues:

5'AGCTCCTGATTCTCA TGTTUGACAGCTTATC...  
3'TCGAGGACTAAGAGTACAAACTGTCTGAATAG...  
(from Primer SEQ. ID NO. 1)

5'AAACATGAGAATCAGGAGCUAAGGAAGCTAAAATG...  
3'TTTGTACTCTTAGTCCTCG ATT CCTTCGATTTTAC...  
(from Primer SEQ. ID NO. 2)

Following UDG, N,N-dimethyl ethylenediamine treatment and heating, the 3' overlaps on each of these 5' end sequences will be:

3' TTTGTA CTCTTAGTCCTCGATTCTTCGATTTTAC... 5' AAGGAAGCTAA AATG...  
(from Primer SEQ. ID NO. 2)

from which it is apparent that when these overhangs are brought into proximity with each other, they are completely complementary:

| (from primer SEQ. ID NO. 1)  
 3' | 5'  
 TCC TT AGCTCCTGATTCT CATGTTT | GACAGCTTA...  
 AGGAA | TCGAGGACTAAGAGTACAAA CTGTCTGAAT...  
 5' | 3'  
 (from primer |  
 SEQ. ID NO. 2)

Therefore, a long overlap of 20 nucleotide bases generated on each of the PCR fragments to be joined by virtue of the specific placement of the dU residue in the primers to prepare the PCR fragments, provides for a highly specific directional joining of these fragments, which is also a stable joint due to the length of those overlaps or overhangs. In fact, it is not necessary to perform a ligase reaction to covalently link the respective single-strand overlapping segments to the rest of the PCR fragments at the joint sites because the strength of hydrogen bonding interactions between the two complementary 3' overhangs is great enough to maintain the joint between the two PCR fragments and prevent them from becoming detached.

As will also be apparent from the above sequences, there are a large number of dT residues which may have been chosen to be replaced by a dU residue during the preparation of the specific primers. Some of the dT residues are nearer the 5' end of the primer sequence and hence would

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have been less favorable as they would have led to the generation of a 3' overhang having less than 20 nucleotide bases. Some of the dT residues are more distal from the 5' end of the primer sequence and hence might be even more favorable than the one exemplified above as they will lead to generation of an even longer 3' overhang.

(2) In a similar fashion to that mentioned above concerning the specific directional joining of the upstream end of the Cm gene to the upstream end of the Tet gene, it was chosen to prepare an internal joint within the Tet gene so that the Tet gene may be generated from two separate fragments, one to be joined as above to the upstream end of the Cm gene, and one as noted below to be joined to the first Tet fragment, in a specific directional manner on one side and to the downstream end fragment on the other side. The primers for these other PCR fragments were specifically designed to provide for these joints. Furthermore, it was chosen to prepare the Cm gene in the form of two separate PCR fragments to be joined to each other (internal joint) and to the Tet gene as above, as well as to the Amp ColEI-ORI region (as below). For all of these connections it was also necessary to prepare all of the necessary primers for synthesis of the various PCR fragments so that each will eventually provide for the desired specific directional joining of all the fragments.

Thus, for the internal Tet gene fusion the following sequence, as obtained from the sequence provided with the commercially obtained pBR322 plasmid, was chosen as the desired linkage region for the two Tet fragments to provide the desired internal linkage.

internal Tet gene region

5'CGATGATCGGCCTGTCGCTTGCGGTATTTCG3'  
3'GCTACTAGCCGGACAGCGAACGCCATAAGC5'

To produce the two Tet gene fragments ( Tet A and Tet B in Fig. 2 ) by PCR procedure and to ensure the above internal connection in a specific

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directional way, the following primers containing the noted dU residues were prepared:

a) Primer SEQ. ID NO. 3, also designated 25595:

Tet gene internal region - sense strand

5'ATCGGCTGTGCTTGCGGTAUTCG3'

b) Primer SEQ. ID NO. 4, also designated 27341:

Tet gene internal region - anti sense strand.

5'ATACCGCAAGCGACAGGCCGAUCATCG3'

For the relative positions of these primers with respect to the completed constructed plasmid and the direction of synthesis from these primers during the PCR procedure, there is shown in Fig. 2 the two primers with arrows indicating their directions.

In an analogous manner to that described above with respect to the Cm upstream - Tet upstream junction, the above primers were used to generate the required PCR fragments having terminal sequences inclusive of the above primer sequences. Following UDG, N,N-dimethylethylenediamine treatment and heating of these PCR fragments, 3' single-stranded overhangs of 22 nucleotide bases are generated on each PCR fragment. As is apparent from the above primer sequences and their directionality, these 3' single-stranded overhangs are completely complementary and hence will readily combine with each other to form the desired Tet gene internal region linkage. Here, too, the nature of the complementary interaction of the 22 base pairs is strong enough to link tightly the two fragments without the necessity for a subsequent ligase reaction.

(3) For the next desired linkage in the plasmid to be constructed, namely, the linkage between the downstream end of the Tet gene and the

downstream end of the Amp-ColEI-ORI region, both from plasmid pBR322, the following sequence was chosen as the desired linkage region for the PCR fragments to be produced containing the above gene regions:

To produce the PCR fragments which will contain the above linkage region to enable specific directional joining between these fragments, the following primers containing the noted dU residues were prepared:

**b) Primer SEQ. ID NO. 6, also designated as 3885:**

For the relative positions of these primers with respect to the completed constructed plasmid and the direction of synthesis from these primers during the PCR procedures, there is shown in Fig. 2 the two primers with arrows indicating their directions.

As noted above for the other fragments, these primers were used to generate the required PCR fragments having terminal sequences inclusive of the above primer sequences. Following UDG, N,N-dimethylethylenediamine treatment and heating of these PCR fragments, 3' single-stranded overhangs of 26 nucleotide bases are generated on each PCR fragment. As is apparent from the above primer sequences and their directionality, these 3' single-stranded overhangs are completely complementary and hence will readily combine with each other to form the desired 3' Amp-ColE1-ORI - 3' Tet gene linkage. There

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too, the nature of the complementary interaction of the 26 base pairs is strong enough to tightly combine the two fragments without the necessity for a subsequent ligase reaction.

(4) The next desired linkage in the plasmid to be constructed was the linkage between the Amp -ColE1-ORI fragment, upstream of the Amp gene and the downstream of the Cm gene. A study of the respective sequences revealed the following as the desired sequence for the linkage region for the PCR fragments to be produced containing the above gene regions:

Amp upstream region	junction point ↓	Cm downstream region
...5' TGCTCACAT GTTCTTT CCTGC GTT	↓	CAGGCGTTTAAGGGCACCAATAAC 3'...
...3' ACGAGTGTACAAGAAAGGACGCAA	↑	GTCC GCAAATTCCTGGTTATTG 5'...

To produce the PCR fragments which will contain the above linkage region to enable specific directional linkage between these fragments, the following primers containing the noted dU residues were prepared:

a) Primer SEQ. ID NO. 7, also designated as 4144:

Amp region	junction point	Cm region
5'ATGTTCTTTCTCCTGCGTT	↑	CAGGCGTTTAAGGGCACCAATAAC 3'

b) Primer SEQ. ID NO. 8, also designated as 27342:

Cm region	junction point	Amp region
5'ACGCCTG	↑	AACGCAGGAAAGAACAUGTG 3'

The relative positions of these primers with respect to the completed constructed plasmid and the direction of synthesis from these primers during the PCR procedures are shown in Fig. 2, with arrows indicating their directions.

As mentioned above, with regard to the other primers, these primers were used to generate the required PCR fragments having terminal

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sequences inclusive of the above primer sequences. Following UDG, N,N-dimethyl ethylene diamine treatment and heating, of these PCR fragments, 3' single-stranded overhangs of 24 nucleotide bases are generated on each PCR fragment. As is apparent from the above primer sequences and their directionality, these 3' single-stranded overhangs are completely complementary and hence will readily combine with each other to form the desired Amp-ColE1-ORI Cm gene linkage. Again, the nature of the complementary interaction of the 24 base pairs is strong enough to facilitate tight binding between the two fragments without the necessity for a subsequent ligase reaction.

(5) The next desired linkage in the plasmid to be constructed was the internal linkage between the two PCR fragments each having part of the Cm gene (Cm A and Cm B fragments in Fig. 2). As noted above, all the sequence information of the internal part of the Cm gene is available with the purchased pACYC184 plasmid. Hence, upon a study of the Cm gene sequence, the following internal Cm sequence was chosen as the desired sequence for the internal joining of the PCR fragments to be produced containing the two Cm gene portions:

internal region of Cm gene (bases 4018-4046 of the pACYC184 map)

5' GGATTGGCTGAGACGAAAAACATATTCTC 3'  
 3' CCTAACCGACTCTGCTTTTGTATAAGAG 5'

To produce the PCR fragments which will contain the above linkage region to enable specific directional linkage between these fragments, the following primers containing the noted dU residues were prepared:

a) Primer SEQ. ID NO. 9, also designated as 27343:

Cm internal region - non-coding strand (anti-sense)  
 5' ATTGGCTGAGACGAAAAACATAUTCTC 3'

b) Primer SEQ. ID NO. 10, also designated as 25596:

Cm internal region - coding strand (sense)  
 5' ATATGTTTTTCGTCTCAGCCAAUCC 3'

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The relative positions of these primers with respect to the completed constructed plasmid and the direction of synthesis from these primers during the PCR procedures are shown in Fig. 2 with arrows indicating their directions.

As mentioned above, with regard to the other primers, these primers were used to generate the required PCR fragments having terminal sequences inclusive of the above primer sequences. Following UDG, N,N-dimethyl ethylenediamine treatment and heating of these PCR fragments, 3' single-stranded overhangs of 23 nucleotide bases are generated on each PCR fragment. As is apparent from the above primer sequences and their directionality, these 3' single-stranded overhangs are completely complementary, and hence will readily combine with each other to form the desired internal Cm gene linkage. Again, the nature of the complementary interaction of the 23 bases pairs is strong enough to facilitate tight binding between the two fragments without the necessity for a subsequent ligase reaction.

It should be mentioned that the selection of the above primer sequences, in particular, those for the joining of two fragments making up a single gene region (internal junction), was not a rigid selection, as any suitable sequence could have been chosen, as long as it provided the desired junction between two specific fragments to be joined. In other words, each of the above primer sequences represents but one possibility from among many which could have been equally suitable. The only constraints in the selection of the primer sequences are that they: (a) will have at least one dU insertion at a position providing for a subsequent 3' overlap on the resulting PCR fragments of at least 15 nucleotides and preferably about 20 nucleotides; (b) they are chosen so as to include in their sequence portions of the sequence of both of the DNA fragments it is desired to join, thereby ensuring a "seamless" joining of the two



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fragments, without any undesired "linker" sequence being inserted into the junction region between the two joined DNA fragments; and (c) that the primer sequences are so chosen to provide for complete complementarity only between the subsequently generated 3' overhangs of two fragments (the specific ends of these fragments) that it is desired to combine, such that when a number of fragments are to be joined in a desired pre-selected order, only the desired junctions will be possible.

For example, in the above primers SEQ. ID NO. 9 and SEQ. ID NO. 10 for the generation of PCR fragments encoding the internal region of the Cm gene to be specifically joined together, the primer sequences may have been chosen from among many possibilities, each having part of the Cm A fragment sequence and part of the Cm B fragment sequence, i.e., each primer covering the desired junction region between the Cm A and Cm B fragments. In this instance, because the junction is an internal one within a single gene region, there is even more flexibility of choice of primer sequence, as the junction point may have been pre-selected anywhere within the Cm gene. Further, the so-chosen primers SEQ. ID NO. 9 and SEQ. ID NO. 10 also have in their sequence numerous dT residues, a number of which (in addition to the exemplified one) may also have been replaced by a dU residue to provide subsequently the desired long 3' overhangs for the purposes of joining the PCR fragments generated from these primers. As the primer sequences were chosen specifically from the Cm gene sequence, there is ensured that once the generated PCR fragments are joined via their 3' overhangs, the joined fragments will contain only the Cm gene sequence, i.e., there is a "seamless" junction.

#### **(ii) Preparation of the PCR fragments**

Taking all of the above into consideration, the following is a brief description of how each fragment of the plasmid of Fig. 2 was prepared

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using all of the various primers for each fragment, and how all the so-produced fragments were linked together:

a) The Amp-ColEI-ORI region fragment was prepared by standard PCR conditions using the above-noted primers SEQ. ID NO. 8 and SEQ. ID NO. 6 (see Fig. 2 for their relative positions and directionality with respect to the completed plasmid), as well as the purchased pBR322 plasmid as the template DNA. The amounts of primer DNA, template DNA and other conditions of the PCR production were as noted hereinabove.

b) The upstream portion of the Tet gene (the Tet A fragment shown in Fig. 2) was prepared by standard PCR conditions using the above-noted primers SEQ. ID NO. 1 and SEQ. ID NO. 4 (see Fig. 2 for their relative positions and directionality with respect to the completed plasmid), as well as the purchased pBR322 plasmid as the template DNA. The amounts of primer DNA, template DNA and other conditions of the PCR procedures were as noted hereinabove.

c) The downstream portion of the Tet gene (the Tet B fragment shown in Fig. 2) was prepared by standard PCR conditions using the above-noted primers SEQ. ID NO. 3 and SEQ. ID NO. 5 (see Fig. 2 for their relative positions and directionality, with respect to the completed plasmid), as well as the purchased pBR322 plasmid as the template DNA. The amounts of primers DNA, template DNA and other conditions of the PCR procedure were as noted hereinabove.

d) The upstream portion of the Cm gene (the Cm A fragment shown in Fig. 2) was prepared by standard PCR conditions using the above-noted primers SEQ. ID NO. 2 and SEQ. ID NO. 9 (see Fig. 2 for their relative positions and directionality, with respect to the completed plasmid), as well as the purchased pACYC184 plasmid as the template

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DNA. The amounts of primer DNA, template DNA and other conditions of the PCR procedures were as noted hereinabove.

e) The downstream portion of the Cm gene (the Cm B fragment shown in Fig. 2) was prepared by standard PCR conditions using the above-noted primers SEQ. ID NO. 10 and SEQ. ID NO. 7 (see Fig 2 for their relative positions and directionality, with respect to the completed plasmid), as well as the purchased pACYC184 plasmid as the template DNA. The amounts of primer DNA, template DNA and other conditions of the PCR procedures were as noted hereinabove.

For PCR, the following conditions and concentrations were used: 10 $\mu$ l of a 0.1 mg/ml solution of primer #1 and primer #2, respectively, were mixed and 180 $\mu$ l of PCR mix as given below added. Then, 1 $\mu$ l of 40ng/ $\mu$ l of DNA template was added, and the PCR carried out at the temperature regime indicated below.

PCR mix:

dNTPs (2.5mM each)	128 $\mu$ l
Buff. x10	160 $\mu$ l
H <sub>2</sub> O	1088 $\mu$ l
Taq DNA Polymerase 5u/ $\mu$ l	6.4 $\mu$ l

Temperature regime

94°C 40sec

40°C 2min

72°C 4min

30 cycles

72°C 5min

6°C infinitely

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Following PCR synthesis of the individual fragments separately, each fragment was then purified by standard agarose-gel purification techniques using the commercially available Bio-Rad "Prep-A-Gene™" DNA purification kit and adhering to the manufacturer's instructions. Following purification, the concentration of the purified fragment DNA was determined by standard procedures using the Pharmacia "Gene-Quant™ RNA/DNA Calculator" and adhering to the manufacture's instructions.

**(iii) Connection of the PCR - produced fragments**

The 5 PCR fragments as produced and purified according to the above-mentioned procedure, were connected to each other in a one-step reaction mixture in a single reaction vessel. This was achieved by mixing the fragments together. Each DNA fragment was in an amount of 0.15 pmol. The volume of the DNA fragments mixture was 9 µl. 10 µl "dU nicking mixture" was added to the DNA fragments mixture. The dU nicking mixture containing 40mM Tris-HCl pH 8.4, 100 mM KCl, 3mM MgCl<sub>2</sub>, 200mM N,N-dimethyl ethylenediamine. 1 µl UDG (in a concentration of 5 U/µl purchased from GibcoBRL), was then added to the reaction, and the mixture was incubated at 37°C for 30 minutes.

Following the above reaction to apurinate the uracil bases from the fragments with UDG and to nick the DNA 3' to the apurinated dU residues with N,N-dimethyl ethylenediamine, thereby preparing the way for dissociation of short single-strands (between 21-26 bases in length, as noted above), the reaction mixture was subjected to conditions to facilitate dissociation of the short nicked single-stranded DNA (oligonucleotides) from the 5' ends of the fragments in order to expose the desired 3' overhangs. These conditions included adding 20 µl Mineral Oil to the reaction mixture (to prevent evaporation) and then heating the reaction mixture to 75°C, at which temperature the mixture was

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incubated for 30 min. At 75°C, the oligonucleotides (short single-strands) upstream of the dU residue at each end of the fragments dissociate from the remaining major portion of the fragments, leaving exposed 3' single stranded overhangs on each end of each fragment.

The next stage was to facilitate the completion of the specific directional joining of the fragments by cooling the above reaction mixture to 37°C (cooling from 75°C at a rate of about 2°C per hour).

(iv) analysis of the products

A 1 µl sample of the above reaction mixture containing the newly constructed plasmid made from joining the 5 separate fragments was used to transform suitable bacterial cells by standard procedures. The mode of transformation was by the preferred standard method of electroporation of *E. coli* DH10B cells. In the transformation procedure 20 µl of electrocompetent "ElectroMax" cells (purchased from Gibco BRL) were mixed with the above 1 µl DNA sample and subjected to electroporation in a commercially available apparatus (BioRad "*E. coli* Pulser Apparatus" set at 1.8 kV and operated according to the manufacturer's instructions).

Following electroporation (transformation) the cells were plated on a standard LB Agar plate containing 100 mM ampicillin (to select for transformants having ampicillin resistance by virtue of having being transformed with a DNA carrying the Amp gene)

The Agar plates were incubated under standard conditions. Over a hundred colonies were obtained and tested further for resistance to chloramphenicol and tetracycline. Thus, colonies found to be resistant to all three antibiotics are indicative of those having received a fully intact constructed plasmid which carries all three resistance genes, namely, the

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desired constructed plasmid (having the desired structure as depicted in Fig. 2).

The results showed that about 10% of the originally obtained ampicillin-resistant colonies were also tetracycline and chloramphenicol resistant. Additional PCR verification tests were carried out on these colonies in order to verify that they carried the required plasmids. Pairs of primers originally used to produce two different fragments were used in a PCR test procedure in which the template DNA was the construct DNA obtained from the transformants. If the fragments had been connected in the correct manner, the PCR product would be equal in size to the sum of the original fragments. The PCR procedure and conditions were the same as noted above under "Materials and Methods". The PCR products were subjected to standard Agarose gel electrophoresis and the resultant bands separated on the gel were analyzed against standard molecular weight markers to determine their approximate size and to determine whether these sizes are the expected ones.

All of the colonies that were subjected to the above PCR test, had constructs that yielded PCR bands having the expected sizes (results not shown).

The above result is a very significant one, as it provides evidence for the first time that it is possible to join correctly 5 separate DNA fragments in a specific directional manner, in a single reaction mixture by an essentially one-step procedure.

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Example 2Improvements in the procedure for the construction of a plasmid  
from 5 separate fragments.

In view of the results set forth in Example 1 above, it was desired to improve the procedure of plasmid construction to obtain a higher yield of fully constructed plasmids from 5 original separate fragments.

In this improved procedure, it was chosen to utilize recently developed technology whereby DNA molecules can be biotinylated and subsequently attached to streptavidin beads to effectively immobilize such biotinylated DNA molecules and remove them from the reaction mixture. Such biotinylation was performed at the terminal adenosine residue for ease of operation. The advantage of this approach is that it removes almost all the oligonucleotides which may interfere with the connection between fragments. Furthermore, it also removes fragments that have two, or even one unexposed terminus, further improving the efficiency of the assembly of fragments.

Using this technology, it was chosen to biotinylate all of the above noted primers SEQ. ID NO. 1, SEQ. ID NO. 2, SEQ. ID NO. 3, SEQ. ID NO. 4, SEQ. ID NO. 5, SEQ. ID NO. 6, SEQ. ID NO. 7, SEQ. ID NO. 8, SEQ. ID NO. 9 and SEQ. ID NO. 10 at their 5' terminal adenosine residues. Biotinylation of the primers is carried out at the time of their synthesis, namely, a biotinylated dA residue is prepared by standard procedures or purchased from a commercial supplier, and is used in the primer synthesis reaction as the last nucleotide, i.e., the 5' terminal nucleotide (primer synthesis using automated apparatus and procedures has the synthesis in the 3'-5' direction). All the other dA residues added during primer synthesis will be normal, non-biotinylated ones. In this way, each primer so synthesized will be biotinylated only at its 5' terminal adenosine residue. As a result, the PCR fragments to be produced with these primers will be 5' biotinylated (two biotinylated 5' adenosine

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residues per fragment produced as each fragment is generated with two primers - see above procedure for the fragment preparation). These biotinylated short strands may be attached to streptavidin-coated beads.

The entire procedure of PCR fragment preparation, was carried out using biotinylated primers which were otherwise identical to those described in Example 1.

In addition, it was also chosen to perform a phosphorothiolation of the 3' ends of each of the primers before use in the PCR procedure, to ensure that the 3' ends of each of the various primers would not be digested by "proof-reading" functions of the high-fidelity DNA polymerases to be used in the PCR procedure. In this way, all of the above primers were also 3' phosphorothiolated during their synthesis before being used in the PCR reactions to generate the various DNA fragments. This phosphorothiolation of the primers being another improvement of the basic method of the invention described in Example 1.

It should, however, be noted that for the purposes of employing high-fidelity DNA polymerases, as noted above, in the PCR reactions, different buffers and other reaction conditions are necessary. Hence, for the purposes of the present PCR reactions, it was chosen to use the same DNA polymerase and PCR reaction conditions as noted above in Example 1. In any event, such modified primers are also capable of being employed with high-fidelity DNA polymerases in accordingly modified PCR reactions.

Thus, once the primers were prepared as above with 5' biotinylation and 3' phosphorothiolation, the PCR fragments were produced by the same procedure as in Example 1, up to and including the step of treating the fragments with UDG and N,N-dimethylethylenediamine and incubating the reaction mixture at 37°C for 30 min. For the subsequent joining of the



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fragments, the reaction was divided at this stage into two separate vessels (one serving as a control).

To the first vessel of 100  $\mu$ l mixture, magnetic streptavidin-coated beads were added (100  $\mu$ l, purchased from MPG) as was 20  $\mu$ l of Mineral Oil to prevent evaporation, and the vessel was then incubated with occasional shaking at 75°C for 30 min. As noted above, at 75°C, the short 5' oligonucleotides upstream of the dU residue at each end of the fragments dissociate from the main portion of the fragments, leaving 3' overhangs. In the presence of the streptavidin-coated beads, these 5' oligonucleotides which are biotinylated are "captured" (i.e., bind tightly,) by the streptavidin molecules and are effectively removed from the "construction mixture", preventing them from competing with the fragment-fragment connections. Following the 30 min incubation at 75°C, the streptavidin beads, which are magnetic, were removed from the reaction vessel by the simple standard procedure of collecting and removing them with a magnet, under suitable conditions so as not to lead to any damage of the DNA fragments in the mixture. Therefore, the short biotinylated 5' oligonucleotides were physically removed from the reaction mixture before the cooling stage, in which complete stabilization of the interacting fragments takes place, to facilitate the final stage of the specific directional connections between the various fragments.

In the second reaction vessel, 100  $\mu$ l H<sub>2</sub>O was added instead of streptavidin-coated beads. This was thus a control experiment, being essentially the same as that described in Example 1 above, in which the 5' oligonucleotides remain in the reaction mixture and can interfere with the fragment-fragment interconnections. All other reaction conditions were as before.

For both of the above reactions, following the 30 min. incubation at 75°C, the slow cooling procedure was carried out, as described in Example 1.

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Likewise, 1  $\mu$ l DNA samples from each of the above two reaction vessels (with and without streptavidin beads treatment) were used to transform the same strain of E. coli cells by the same procedure as detailed in Example 1.

The results of the E. coli transformations revealed that for the streptavidin-treated reaction mixture, 480 ampicillin-resistant colonies were obtained, of which 35% were chloramphenicol- and tetracycline-resistant. In contrast, for the non-streptavidin-treated reaction mixture, only 160 ampicillin-resistant colonies were obtained, of which only about 10% were also resistant to chloramphenicol and ampicillin (this being essentially the same result as obtained for the basic procedure set forth in Example 1).

The above significantly greater number of completely constructed desired plasmids obtained by biotinylation-streptavidin treatment indicates that the free released 5' oligonucleotides do interfere with the connections between the various fragments, and their removal by the biotinylation-streptavidin treatment is desirable.

The use of the Biotin-Streptavidin system, for removing of the oligonucleotide generated by nicking at the apurinated site, is for illustrative purposes only. It can be done by several different methods, as for example the use of commercially available kits for removing oligonucleotides (see Example 3 hereinbelow).

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### Example 3

#### Preparation of a circularized plasmid by the directional connection of 8 fragments constituting the plasmid

##### Overview

The plasmid to be constructed was designed to consist of eight fragments, each to be prepared separately by PCR amplification and then joined in a specific directional fashion to provide a circularized plasmid as the end-product. This example is similar to example 1, the difference being the number of fragments the plasmid was constructed of.

In Fig. 3, there is shown schematically the plasmid that was designed and produced by the method of the present invention. This plasmid carries four independent antibiotic resistance genes, for resistance to ampicillin (Amp<sup>r</sup> gene, or hereinafter Amp); tetracycline (Tet<sup>r</sup> gene, or hereinafter Tet); chloramphenicol (Cm<sup>r</sup> gene, or hereinafter Cm) and kanamycin (Kn<sup>r</sup> gene, or hereinafter Kn). The plasmid also carries the ColEI origin of replication (ColEI-ORI), which in this specific instance is situated next to the Amp gene. Hence, such a plasmid is capable of being replicated in a host cell and will endow the host cell with resistance to all four types of antibiotic.

Accordingly, it is possible to readily select for those host cells transformed by this plasmid by growing the transfected cells in the presence of one of the antibiotics and then to screen for the resistance to the other antibiotics. In order to verify that mis-connections did not occur, additional verification tests may be carried out. One such test carried out included testing transformed colonies by restriction enzyme analysis. DNA was prepared from a number of transformed colonies and checked by restriction enzyme analysis.

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This constitutes the first known disclosure of a successful, specific directional connection of eight independent PCR fragments to form a single active plasmid construct, and this by an essentially one-step procedure in accordance with the present invention.

For this construction of eight fragments, the following was carried out:




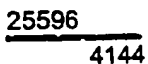

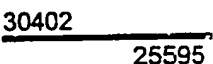

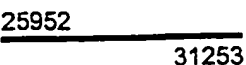
**(i) Preparation of the specific primers for the PCR amplification.**

As shown in Fig. 3, and as outlined above, it was desired to construct a circularized plasmid having four regions:

- (a) a Tet region.
- (b) an Amp + ColEI-ORI region.
- (c) a Cm region.
- (d) a Kn region.

The order of the connection is depicted in figure 3.

To achieve this task, eight separate fragments were designed:

Name of fragment	Size(bp)	plasmid	Location
AmpA 	574	pBR322	3603-4159
AmpB 	1171	pBR322	2460-3624
CmA 	481	PACYC184	4021-240
CmB 	293	PACYC184	3768-4043
TetA 	862	pBR322	1-848
TetB 	631	pBR322	827-1449
KnA 	569	PACYC177	1809-2368
KnB 	652	PACYC177	2349-2991

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Written are the names of the fragments, their sizes, the plasmid from which they were PCR amplified, the exact location of the site on the original plasmid and the numbers of the primers that were used to amplify the fragments. The full sequence and maps of the various regions of these plasmids are known and can be accessed from GenBank database under accession Nos. J01749 (pBR322), X06403 (pACYC184) and X06402 (pACYC177).

The **AmpB** fragment includes the 5' part of the Amp fragment and the ColEI-ORI sequence. The **AmpA** fragment includes the 3' part of the Amp fragment. The **CmA** fragment includes the 5' part of the Cm fragment and the **CmB** fragment includes the 3' part of the Cm fragment. The **TetB** fragment includes the 5' part of the Tet fragment and the **TetA** fragment includes the 3' part of the Tet fragment. The **KnA** fragment includes the 5' part of the Kn fragment and the **KnB** fragment includes the 3' part of the Kn fragment.

As in example 1, each primer consists of two regions: a 3' region complementary to the DNA to be amplified, and a 5' region complementary to the fragment it should be connect to.

Using standard automated procedures to produce polynucleotide oligomers (Applied Biosystems, U.S.A.), the following primers were synthesized:

In all the cases below, the (-) in the middle of the sequence represents the junction between the regions of the original DNA.

#### Primers for the amplification of the AmpA fragments

Primer SEQ. ID NO. 11, also designated as 241365:

internal Amp region

5'ATTGCTGCAGGCATCGTGGTGCA 3'

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Primer SEQ. ID NO. 6, also designated as 3885:

Cm region                      Amp region  
5'AGCGTTGGGTCCTGGCCA - AAGAGTAUGAGTATTCAACA 3'

Primers for the amplification of the AmpB fragments

Primer SEQ. ID NO. 8, also designated as 27342:

Cm region                      Amp region  
5'ACGCCTG - AACGCAGGAAAGAACAUGTG 3'

Primer SEQ. ID NO. 12, also designated as 241366:

                    internal Amp region  
5'ACACCACGATGCCTGCAGCAAUGG 3'

Primers for the amplification of the CmA fragments

Primer SEQ. ID NO. 13, also designated as 40122:

                    Kn region                      Cm region  
AGG CCT GGT ATG AGT C - TCA GGA GCU AAG GAA GCT AAA ATG

Primer SEQ. ID NO 9, also designated as 27343:

                    Cm region  
5'ATTGGCTGAGACGAAAAACATAUTCTC 3-

Primers for the amplification of the CmB fragment

Primer SEQ. ID NO. 10, also designated as 25596:

                    Cm region  
5' ATATGTTTTTCGTCTCAGCCAAUCC 3'

Primer SEQ. ID NO. 7, also designated as 4144:

                    Amp region                      Cm region  
5'ATGTTCTTTCTCCTGCGTT - CAGGCGUTTAAGGGCACCAATAAC 3-

Primers for the amplification of the TetA fragment:

Primer SEQ. ID NO. 4, also designated 27341:

                    Tet region  
5'ATACCGCAAGCGACAGGCCGAUCATCG 3'

Primer SEQ. ID NO. 14, also designated as 36176:

                    Kn antisense                      Tet sense  
5'ACGTGGCTTTGTTG - TTCTCATGUTTGACAGCTTATC 3'

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Primers for the amplification of the TetB fragment:

Primer SEQ. ID NO. 5, also designated as 30402:

Amp region                      Tet region  
5'ATACTCTT - TGGCCAGGACCCAACGCUGCCC 3'

Primer SEQ. ID NO. 3, also designated as 25535:

Tet region  
5'ATCGGCCTGTCGCTTGCGGTAUTCG 3'

Primers for the amplification of the KnA fragment:

Primer SEQ. ID NO. 15, also designated as 31254:

Tet region                      Kn region  
5'ACATGAGAA - CAACAAAGCCACGUTGTGTCTC 3'

Primer SEQ. ID NO. 16, also designated as 25953:

Kn region  
5'AGACGAAATACGCGATCGCUGTTAA 3'

Primers for the amplification of the KnB fragment:

Primer SEQ. ID NO. 17, also designated as 25952:

Kn region  
5'AGCGATCGCGTATTTTCGTCUCGCTC 3'

Primer SEQ. ID NO. 18, also designated as 31253:

Cm region                      Kn region  
5'AGCTCCTGA - GACTCATACCAGGCCUGAATCG 3'

(ii) Preparation of the PCR fragments

The PCR was carried out as in Examples 1 and 2. Following PCR synthesis of the individual fragments, each fragment was purified by standard agarose-gel purification techniques using the commercially available Bio-Rad "Prep-A-Gene™" DNA purification kit and adhering to the manufacturer's instructions. Following purification, the

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concentration of the purified fragment DNA was determined by standard procedures.

(iii) Connection of the PCR-produced fragments

The eight PCR fragments were connected to each other in a one-step reaction mixture in a single reaction vessel. This was achieved by mixing the fragments together in a 25 $\mu$ l reaction mixture that included: 0.15 pmol of each fragment, 2.5 $\mu$ l buffer (200mM Tris-HCl pH 8.4, 500 mM KCl, 15mM MgCl<sub>2</sub>), 2.5  $\mu$ l of 1M N,N-dimethyl ethylenediamine and 6.25 units of UDG (GibcoBRL). The mixture was incubated at 37°C for 4 hours and then transferred to 70°C for 5 minutes to facilitate dissociation of the short nicked single-stranded DNA from the 5' ends of the fragments (as explained in example 1). After dissociation of the short nicked single-stranded DNA, the DNA was cleaned from them with "QIAquick PCR purification kit" (QIAGEN) adhering to the manufacturer's instructions. Before adding the first buffer of the kit, 200  $\mu$ l of hot (70°C) buffer (20mM Tris-HCl pH 8.4, 50 mM KCl, 1.5mM MgCl<sub>2</sub>) was added in order to minimize the reannealing of the short nicked single-stranded DNA. This was carried out instead of the biotin-method described in Example 2. The DNA was eluted in 30 $\mu$ l of sterilized water. 27 $\mu$ l of the DNA mixture was incubated with 3 $\mu$ l of buffer (200mM Tris-HCl pH 8.4, 500 mM KCl, 15mM MgCl<sub>2</sub>) in a water-bath at 70°C. The bath was shut down and the temperature was slowly decreased to 37°C. This allows for the joining between the complementary overhangs, while minimizing illegitimate connections.

(iv) analysis of the construct

A 1  $\mu$ l sample of the above DNA containing the newly constructed plasmid, made by joining the 8 separate fragments, was used to



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transform E. coli DH10B cells by electroporation (as detailed in example 1).

Following electroporation (transformation) the cells were plated on LB Agar plate containing 100 mM ampicillin (to select for transformants having ampicillin resistance by virtue of having been transformed with a DNA carrying the Amp<sup>r</sup> gene). On the day after, three colonies were picked and checked for resistance to chloramphenicol, tetracycline and kanamycin by plating them on Agar plates containing the appropriate antibiotics.

The results showed that the three colonies were resistant to all the four antibiotics. The colonies were further checked by restriction enzyme analysis and proved to be correct (data not shown).

The above result is very significant, since it proves that it is possible to correctly join 8 separate DNA fragments in a specific directional manner, in a single reaction mixture by an essentially one-step procedure.

The examples above demonstrate that in accordance with the present invention, it is possible for the first time to construct a fully active plasmid molecule from numerous (e.g., 5, 8) separate fragments in a simple, essentially one-step process, and even under less favorable conditions (without biotinylation-streptavidin treatment), this process is feasible, producing fully constructed desired plasmid molecules.

The above examples have been provided for the purpose of illustration only. As will be appreciated by the skilled person, the invention is not

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limited to any particular conditions, except as defined in the appended claims.

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SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

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(ii) TITLE OF INVENTION: Method for joining DNA  
fragments

(iii) NUMBER OF SEQUENCES: 18

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version  
#1.25 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AGCTCCTGAT TCTCATGTTU GACAGCTTAT C

31

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AAACATGAGA ATCAGGAGCU AAGGAAGCTA AAATG

35

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATCGGCCTGT CGCTTGCGGT AUTCG

25

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## (2) INFORMATION FOR SEQ ID NO: 4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ATACCGCAAG CGACAGGCCG AUCATCG

27

## (2) INFORMATION FOR SEQ ID NO: 5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ATACTCTTTG GCCAGGACCC AACGCUGCCC

30

## (2) INFORMATION FOR SEQ ID NO: 6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid

- 60 -

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AGCGTTGGGT CCTGGCCAAA GAGTAUGAGT ATTCAACA

38

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ATGTTCTTTC CTGCGTTCAG GCGUTTAAGG GCACCAATAA C

41

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

ACGCCTGAAC GCAGGAAAGA ACAUGTG

27

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ATTGGCTGAG ACGAAAAACA TAUTCTC

27

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ATATGTTTTT CGTCTCAGCC AAUCC

25

(2) INFORMATION FOR SEQ ID NO: 11:

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## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

ATTGCTGCAG GCATCGTGGT GUCA

24

## (2) INFORMATION FOR SEQ ID NO: 12:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

ACACCACGAT GCCTGCAGCA AUGG

24

## (2) INFORMATION FOR SEQ ID NO: 13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid



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(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

AGGCCTGGTA TGAGTCTCAG GAGCUAAGGA AGCTAAAATG

40 .

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ACGTGGCTTT GTTGTCTCA TGUTTACAG CTTATC

36

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

- 64 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

ACATGAGAAC AACAAAGCCA CGUTGTGTCT C

31

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

AGACGAAATA CGCGATCGCU GTTAA

25

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

AGCGATCGCG TATTCGTCU CGCTC

25

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(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

AGCTCCTGAG ACTCATACCA GGCCUGAATC G

31

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Claims

1. A method for the simultaneous multi-DNA fragment assembly of two or more double-stranded DNA fragments produced by a primer extension reaction, particularly the polymerase chain reaction (PCR), comprising the steps of:

- (a) providing for each DNA fragment to be joined to a second DNA fragment and optionally to a third DNA fragment, a pair of primers for the primer extension procedure thereof, wherein one of said two primers has a portion which is complementary to one terminal portion of the strand of said fragment, and the second of said two primers has a portion which is complementary to the other terminal portion of the strand of said fragment, and wherein the second portion of at least one of the said primers is complementary to the terminal portion of a different fragment to be joined to said first fragment in a specific positioned relationship;
- (b) providing, in each of said primers, at least one dU residue or the like in place of at least one dT residue of the original primer sequence, the first of said at least one dU residue(s) being positioned at least 15 nucleotides from the 5' end of said primer;
- (c) producing each said DNA fragment by the primer extension reaction procedure;
- (d) carrying out in any suitable order the steps of: 1. mixing the produced DNA fragments 2. adding the enzyme UDG to apurinate said dU residue and a second enzyme or a compound such as N,N-dimethyl ethylenediamine to generate single-strand nicks at said apurinated dU residue, said UDG and second enzyme or compound being in amounts sufficient thereby to generate a nick at the end of substantially all fragments, and dissociating the nicked oligonucleotides which are formed at the 5' end or ends of the fragments, and

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- (e) providing suitable conditions and allowing sufficient time for the specific joining of said fragments to generate a multi-DNA fragment assembled product.
2. A method according to claim 1, wherein the DNA mixing step is carried out before the UDG enzyme addition step.
  3. A method according to claim 1, wherein the UDG enzyme addition step is carried out before the DNA mixing step.
  4. A method according to claim 1-3, wherein step (e) includes the removal of the nicked oligonucleotides from the reaction.
  5. A method according to claim 4, wherein at least some of said primers for the primer extension production of said DNA fragments are biotinylated at their 5' ends, and wherein when said enzyme UDG and said second enzyme or compound are added to said mixture of fragments, there is also added streptavidin, either free or bound to beads, whereby single-stranded oligonucleotides containing at their 5' end a biotin moiety generated following the formation of said single-strand nicks at said apurinated dU residues are bound via a biotin-streptavidin connection and are effectively removed from the joining reaction.
  6. A method according to claims 1-5, wherein said 3' complementary overhangs on said DNA fragments are generated by the addition to said fragments, the enzyme UDG to apurinate said dU residue and a second enzyme selected from endonuclease III (Endo III) or endonuclease IV (Endo IV) to generate single-stranded nicks at said apurinated dU residue to provide said 3' overhangs.
  7. A method according to claims 1-5, wherein said 3' complementary overhangs on said DNA fragments are generated by the addition to said fragments of the enzyme UDG to apurinate said dU residue and of a

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compound suitable to generate single-strand nicks at said apurinated dU residue to provide said 3' overhangs.

8. A method according to claim 7, wherein the compound suitable to generate single-strand nicks at the apurinated dU residue to provide the 3' overhangs is N,N-dimethyl ethylenediamine.

9. A method according to any one of claims 1-8, wherein the joining of the fragments via the 3' complementary overhangs is facilitated *in vitro* by:

(a) heating the reaction mixture to a temperature suitable to allow dissociation of the oligonucleotides at the termini of the fragment after said apurination of the dU residue and generation of nicks at the apurinated dU residue, and incubating said mixture;

(b) keeping the mixture of (a) at a temperature suitable to maximize correct connections between complementary overhangs; and

(c) optionally, adding ligase to said mixture to facilitate covalent joining of the DNA strands.

10. A method according to any one of claims 1-9, wherein after the joining of the fragments via the 3' complementary overhangs is facilitated *in vitro*, the DNA is used to transform and/or transfect host cells.

11. A method according to any one of claims 1-9, wherein the covalent connection of the fragments via the 3' complementary overhangs is facilitated *in vivo*, by the host cells that are being transformed and/or transfected by the mixture of fragments.

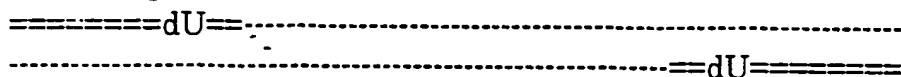
12. A method according to any one of claims 1-11, wherein one or more of said DNA fragments to be joined is a mutant fragment having been subjected to site-directed mutagenesis or mutagenic PCR during its preparation.

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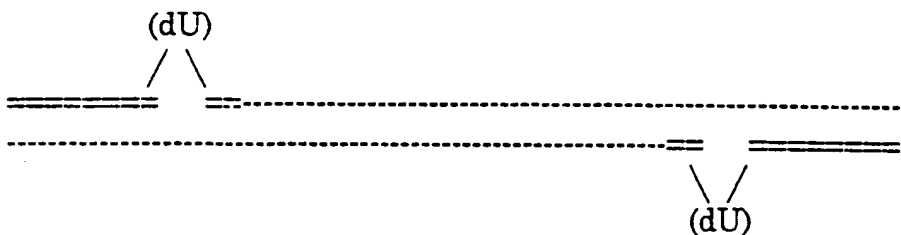
13. A primer for use in a method according to any one of claims 1-12 comprising at least one dU residue in place of at least one dT residue, the first of said at least one dU residue(s) being positioned at least 15 nucleotides from the 5' end of the primer.
14. A primer according to claim 13, wherein said primer is biotinylated at its 5' end.
15. An assembled DNA construct whenever prepared by a method according to any one of claims 1-12, and wherein said construct has been assembled from the joining together of two or more DNA fragments.
16. An assembled DNA construct according to claim 15, wherein said construct is in the form of a linear DNA molecule.
17. An assembled DNA construct according to claim 15, wherein said construct is in the form of a closed circular DNA molecule.
18. A method according to claim 1 wherein one or more of the DNA fragments to be joined comprise sequences coding for one or more proteins selected from the group including cytokines, hormones, and enzymes.
19. A method according to claim 18 wherein the enzymes are selected from the group consisting essentially of citrate synthases, polyketide synthases, and succinyl-CoA-synthetase.
20. A DNA fragment comprising an overhang of at least 15 nucleotides or an end portion suitable to be converted into such an overhang.
21. A DNA fragment as claimed in claim 20, for use in the method of claim 1.

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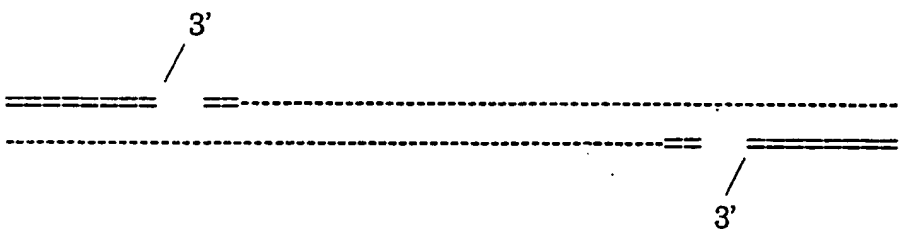
A. PCR fragment with a single dU residue that had been incorporated into each primer



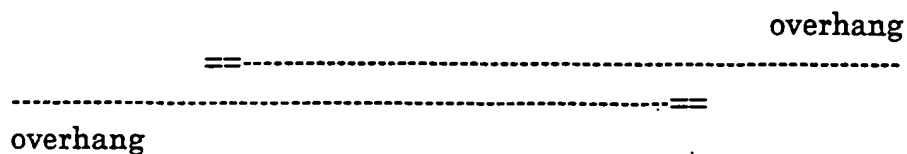
B. UDG a-purinates the dU residues



C. N,N dimethylethylenediamine nicks the a-purinated residues



D. Heating disconnects the nicked oligonucleotides from the double-stranded DNA molecule



E. Slow cooling allows fragments with complementary termini to interconnect

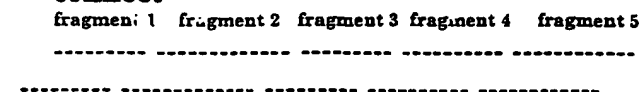


Fig. 1



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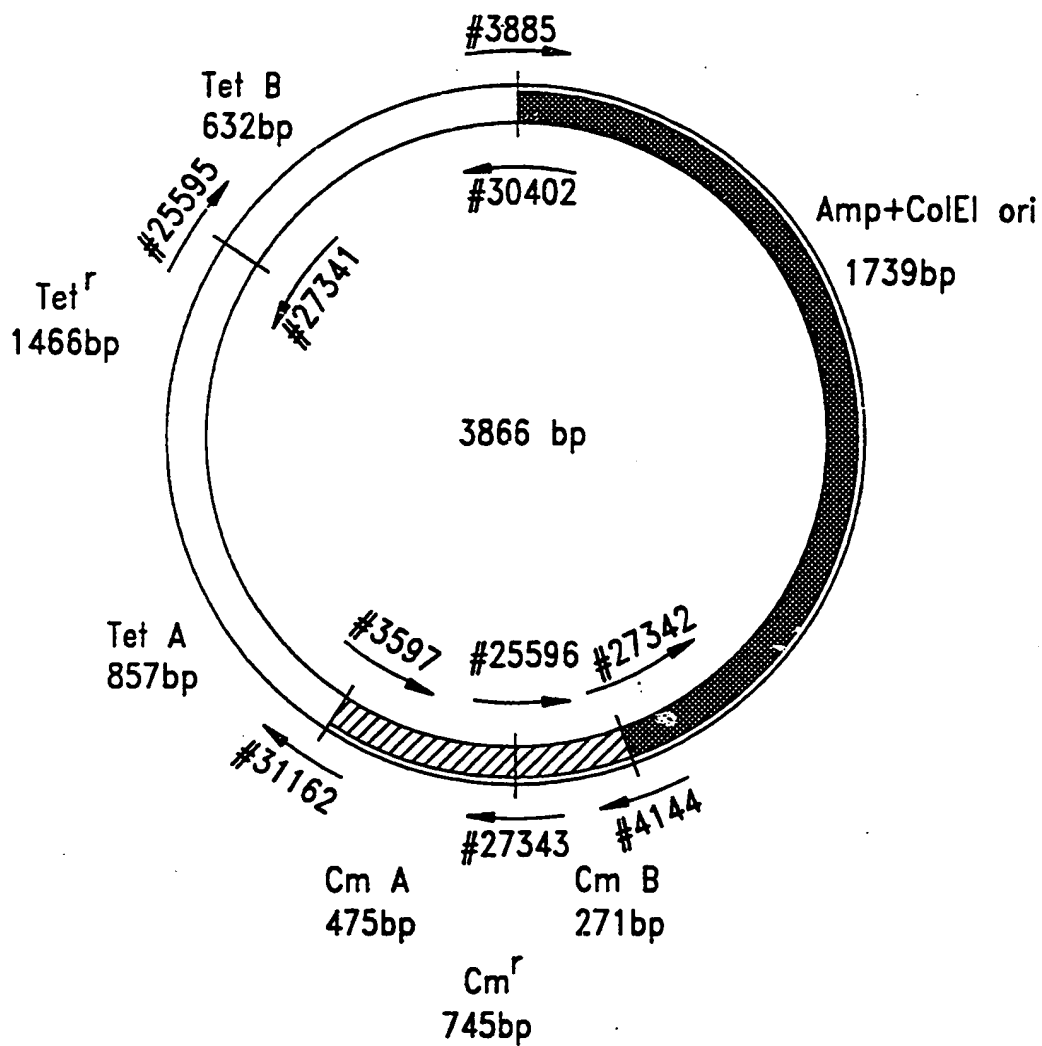


Fig. 2

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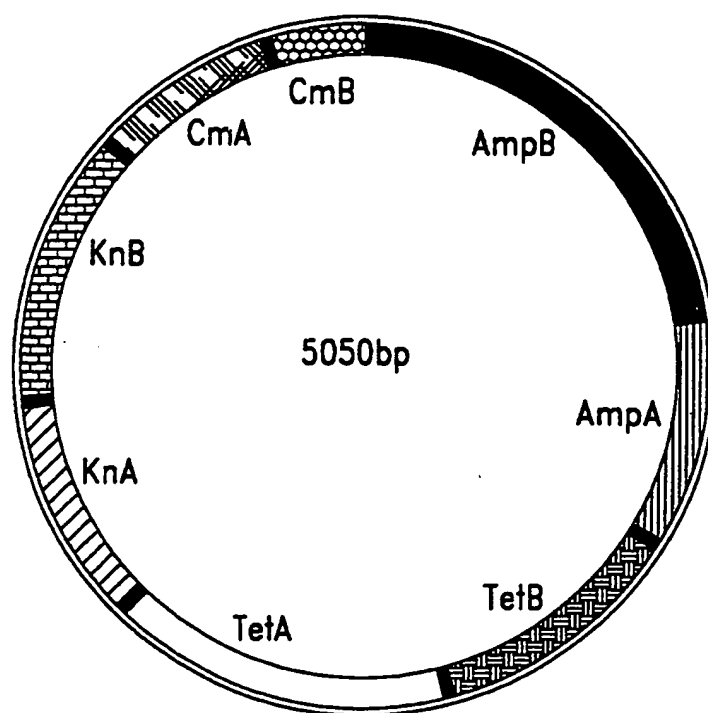


Fig. 3

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/IL 98/00094

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/10 C12Q1/68 C12N15/66

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>BOOTH P.M. ET AL.: "Assembly and cloning of coding sequences for neurotrophic factors directly from genomic DNA using polymerase chain reaction and uracil DNA glycosylase"</p> <p>GENE., vol. 146, no. 2, 1994, AMSTERDAM NL, pages 303-308, XP002071998</p> <p>see the whole document</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	1-21

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

4 August 1998

Date of mailing of the international search report

17/08/1998

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Authorized officer

Panzica, G

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/IL 98/00094

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SMITH C. ET AL.: "Generation of cohesive ends on PCR products by UDG-mediated excision of dU, and application for cloning into restriction digest-linearized vectors" PCR METHODS & APPLICATIONS., vol. 2, no. 4, May 1993, ING HARBOR LABORATORY PRESS US, pages 328-332, XP002071999	20
Y	see the whole document	1-3,6,7, 9,13, 15-18,21
X	EP 0 385 410 A (CANON KK) 5 September 1990	20
Y	see abstract  see figure 1 see page 7, line 45 - page 13, line 55 see examples 1-4,8-13	1-3,6,7, 9,13, 15-18,21
X	RASHTCHIAN A. ET AL.: "URACIL DNA GLYCOSYLASE-MEDIATED CLONING OF POLYMERASE CHAIN REACTION-AMPLIFIED DNA: APPLICATION TO GENOMIC AND cDNA CLONING" ANALYTICAL BIOCHEMISTRY, vol. 206, no. 1, 1 October 1992, pages 91-97, XP000311343 see the whole document	20
X	NEWTON C R ET AL: "PCR" 1994, BIOS SCIENTIFIC PUBLISHERS, OXFORD GB XP002072707 see page 59 - page 70	20
A		1,12
A	WO 93 18175 A (LIFE TECHNOLOGIES INC) 16 September 1993 cited in the application see the whole document	1,12
A	EP 0 456 304 A (EASTMAN KODAK COMPANY) 13 November 1991 see the whole document	1,5,14
X,P	WATSON D.E., BENNETT G.N.: "Cloning and assembly of PCR products using modified primers and DNA repair enzymes" BIOTECHNIQUES., vol. 23, no. 5, 1997, NATICK US, pages 858-860, 862, 864, XP002071997 see the whole document	1,4,6,7, 9,10,13, 15-18, 20,21

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IL 98/00094

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